APPARATUS AND METHOD FOR STORING PROTEINS

Field of the Invention

The invention relates to an apparatus and a method for the storage of proteins.

Prior Art

One of the problems associated with the long-term storage of proteins is that they lose their biological properties over time as the molecule is degraded. Prior art methods of storing proteins have been developed to overcome this problem.

For example, Japanese Patent Application JP-A-63-092671 (Kanebo) teaches a method for the storage of proteins in which fibroin or collagen is dissolved in an aqueous solution. A hydrolysing enzyme is added to the solution followed by a chelating agent. The pH of the mixture is adjusted in the range 4.5 to 7.5 and the solution filtered through a filter having pores of around 1μm or less. Finally the solution is dried to give a polymer with an average degree of polymerisation in the range of 200-600.

It would be desirable, however, to be able to store the protein in its natural state and without enzyme treatment or treatment with chelating agents.

In nature, several methods are known for the storage of proteins. Natural silks are fine, lustrous filaments produced by the silkworm Bombyx mori and other invertebrate species from a stored protein dope or feedstock. The silks offer advantages compared with the synthetic polymers currently used for the manufacture of materials and their properties seem to be substantially unaffected by long-term storage of the protein dope within the organism's gland. For example, the tensile strength and toughness of the dragline silks of certain spiders can exceed that of Kevlar™ fibre, the toughest and strongest man-made fibre. Spider dragline silks also possess high thermal stability. Many silks are also biodegradable and do not persist in the environment. They are recyclable and are produced by a highly efficient low pressure and low temperature process using only water as a solvent. The natural spinning process is remarkable in that an aqueous solution of

protein is converted into a tough and highly insoluble material. The process in spiders and silkworms produce silk threads has been outlined in an article "Liquid crystalline spinning of spider silk" by Vollrath and Knight, Nature, vol. 410, 29 March 2001, pages 541-8. The authors note that lessons are to be learnt from the manner in which spiders and silkworms store their protein dope molecules and extrude them into strong threads. The article reviews the evidence that the natural spinning mechanism in spiders involves an addition of hydrogen ions and potassium ions and the removal of sodium ions as the spinning dope passes down the spinning duct.

In an article by Knight and Vollrath "Biological Liquid Crystal Elastomers", Trans. Phil. R. Soc. B. 357, 155-163 (2002), the authors point out that the assembly of spidroin and fibroin proteins to form tough threads depends on the fact that the chief structural proteins of silks, fibroins in silkworms and spidroins in spiders, are amphiphilic repetitive block copolymers of the type ABAB, where A represents a hydrophobic block and B a less hydrophobic one.

Spidroin and fibroin are found in two states: The first state is a safe storage state in which the extremely long protein chains are thought to be folded into short, rather compact rod-shaped molecules. The fibroin or spidroin proteins in this first state have a predominantly random coil and/or helical secondary structure. The second state is a solid state with a predominantly beta crystalline secondary structure. This second state is a nanofibrillar composite, containing a high packing fraction of very long nanofibrils approximately 5 nm in diameter. The nanofibrils are oriented substantially parallel to the long axis of the tough thread and are thought to contain all or most of the beta crystallites. The beta crystallites have a width of about 5nm and are arranged substantially parallel to the long axis of the nanofibrils. Small quantities of a less crystalline and more disordered material are thought to form the matrix between the nanofibrils.

The first, storage state is metastable. Its conversion to the second nanofibrillar state is thought to involve both an aggregation of the molecules and a change in conformation of the secondary structure. A change in conformation is thought to occur in the most hydrophobic of the block types of the repetitive block co-polymer which transform from a 'random coil' / alpha helical to the beta crystalline structure. At least five factors are thought to be involved in the aggregation and conformation transition which forms tough

threads in vivo: a reduction of the pH; the addition of potassium ions to the protein; the removal of sodium ions from the protein; the loss of water and the application of mechanical strain to the forming threads. In addition the conversion may be promoted by the secretion of polyols and surfactants. Fine longitudinal ridges with low surface energy in the final part of the spinning duct lining in spiders may help to promote the conversion of the protein dope to a solid thread. Once short nanofibrils have formed these may act as seeds initiating further aggregation and conformation change of the protein, thus enhancing the overall rate of the transition.

If solutions of the first storage state of fibroin or spidroin are left untreated they transform spontaneously into the insoluble beta crystalline state thus prematurely forming insoluble flocs or gels that cannot be extruded or spun into useful threads. The transformation can be remarkably rapid, usually taking 1-3 days for completion. This is a problem when extruding or spinning threads from silk proteins. Bacterial proteolysis may play a part in this transformation in vitro by cutting proteins into shorter peptides that change in conformation or crystallise more readily. Alternatively proteolysis may promote the transformation by removing protective domains which inhibit aggregation or conformation change. Transformation of a native fibroin or spidroin solution to the insoluble form can also be promoted by seeding with material already transformed into the beta state. Freezing or mechanically shearing native solutions will also result in transformation to the beta crystalline state. Regenerated fibroin and spidroin solutions prepared by dissolving silk threads in a chaotropic agent such as lithium bromide, lithium thiocyanate, sodium thiocyanate, calcium chloride, calcium nitrate also gradually undergo an analogous formation of a floc or gel when the chaotropic agent is removed by dialysis. This again presents a problem when seeking to spin or extrude materials from regenerated silks.

The first storage state is found in the posterior and middle divisions of the gland in silkworms and in the analogous A-zone in spiders. In these regions of the respective glands the protein is stored at remarkably high concentrations (20-40% w/v). In spiders, the protein is thought to be stored as a highly viscous liquid crystalline sol that persists through the first, second and most of third limb of the silk gland's duct. In silkworms, the protein is stored as a gel within the posterior and middle division of the silk gland in newly moulted final instar silkworms, but is transformed into a sol in the duct (anterior

part of the anterior division) shortly before the protein is spun. Thereafter the transformation from gel to sol propagates backwards down the secretory pathway to enable the stored material in the middle and posterior divisions to flow forward down so that it can be drawn down to a filament in the anterior (distal part) of the duct...

Summary of the Invention

The object of the invention is to provide an apparatus and method for the storage of proteins.

These and other objects of the invention are solved by providing an apparatus for the storage of a protein comprising a first compartment for storing the protein and a second compartment for storing an alkaline buffer. In the preferred embodiment of the invention, the second compartment contains an alkaline buffer containing calcium chloride. The second compartment is in fluid (i.e. liquid or gaseous) communication with the first compartment. The protein stored in the first compartment is therefore in an alkaline condition containing calcium ions under which conditions it is considerably more stable than untreated spidroin or fibroin solutions removed directly from the organisms' glands or prepared from by dissolving spider or silkworm silk in chaotropic agents. The decomposition or premature formation of the beta-sheet form formation of the beta state of the protein is thereby greatly retarded.

In one embodiment of the invention, the alkaline buffer is selected from the group of alkalis consisting of ammonia, ammonium acetate, ammonium formate, ammonium citrate, Tris/HCl, HEPES, PIPES, sodium carbonate, potassium carbonate, sodium phosphate, potassium phosphate or a mixture of these.

In an embodiment, sodium azide is added to the protein in addition to the alkaline buffer. Alternatively, phenyl thiourea, sodium cyanide or potassium cyanide might be added.

Also in an embodiment, 100-700 mM of calcium ions are added to the alkaline buffer, preferably as chloride. Under these circumstances, the alkaline buffer may not contain carbonate or phosphate ions.

When calcium ions are added to the alkaline buffer, they cause concentrated solution of silk proteins or their analogues to gel. The gel produced in this way is considerably more stable than the sol state solutions of silk proteins. The gel state induced in this way can be converted back to a sol state before extruding, spinning or otherwise forming the silk. This can be achieved by dialysis against a dialysis solution containing 20-100 mM ethylenediamine tetraacetic acid (EDTA) solution adjusted to pH 7.0-7.8 with ammonia or sodium hydroxide solution. This treatment removes calcium ions. Alternatively, calcium ions can be removed by dialysis against ion-free water. In either case polyethylene glycol or another water soluble polymer can be added to the dialsyant to maintain or increase the protein concentration by reverse dialysis.

In the preferred embodiment at least part of the inner surface of the walls of the first storage compartment are formed from or coated with a material with low surface energy such as polytetrafluoroethylene (PTFE), silane, nylon, polyethylene or polycarbonate which also helps to prevent premature formation of the beta sheet form of the protein.

In the apparatus the alkaline buffer is in either a gaseous form or a solution. If it is used in a gaseous form it can be applied directly to the surface or surfaces of the protein solution or can be allowed to diffuse into it through a porous or semi-permeable surface. If the alkaline buffer is used in solution it can be separated from the protein by a porous or semipermeable membrane or a separate flow of buffer solution can be applied to the flow of protein solution and subsequently lead away from the surface of the protein solution. If the alkaline buffer is in a gaseous form it can diffuse directly into the protein solution to render it alkaline. If the alkaline buffer is separated from the protein by a porous or semipermeable membrane, then both buffer and calcium ions it may contain can diffuse through the said membrane into the protein solution.

In a further embodiment of the invention, the protein is mixed directly with an alkaline solution as this helps keep the protein in a sol state. Calcium ions can be added directly to the alkaline solution.

In one preferential embodiment of the invention, sodium azide is added to a buffer with a pH greater than 7.4. to give a final concentration in excess of 0.0001 M.

The stored protein can be either a natural protein obtained, for example, by the dissection of an animal, or recombinant protein obtained by genetically engineering or a regenerated silk solution prepared by dissolving silkworm or silk fibres in a chaotropic agent that is subsequently removed by dialysis, or a mixture of the aforementioned proteins or protein analogues. The invention has been found to be useful in the storage of fibroin or spidroin proteins or homologues thereof or regenerated solutions of fibroin and/or spidroin.

More generally, the proteins stored are repetitive amphiphilic block co-polymeric proteins or protein analogues both containing charged groups and which are prepared by chemical synthesis or genetic engineering

The object of the invention is also solved by providing a method for the storage of a protein comprising a first step of placing the protein in a first storage compartment. In a second step, the protein is exposed to an alkaline buffer (preferably containing calcium ions and sodium azide) for a period of time. In a third step, the protein is maintained in an alkaline environment in the first storage compartment.

This provides a long-term storage solution for silk proteins or their analogues and regenerated silk solutions.

Description of the Drawings

Fig. 1 shows a schematic diagram of a first embodiment of an apparatus suitable for the storage of proteins.

Fig. 2 shows a schematic diagram of a second embodiment of an apparatus suitable for the storage of proteins.

Fig. 3 shows the results of effect of alkaline buffer and sodium azide on storage times for concentrated native fibroin solutions.

Detailed Description of the Invention

Fig. 1 is a schematic diagram illustrating a first embodiment of an apparatus 10 suitable for storage of a protein 20. The apparatus 10 has a protein storage compartment 30 in which the protein 20 is placed. The protein storage compartment 30 is connected by means of a pipe or tube 35 to an alkali storage compartment 40. The alkali storage compartment 40 stores an alkaline solution 50. The protein storage compartment 30 has preferably in part or substantially all inner walls which are made from or coated with a material with a low surface energy such as polytetrafluoroethylene (PTFE), polyethylene or polycarbonate.

The protein 20 in the protein storage compartment 30 can be a natural protein that is obtained, for example, by the dissection of an animal. Examples of such natural proteins include, but are not limited to, spidroin protein obtained from the major ampullate gland of spiders of the genus Nephila or fibroin protein obtained from Brombyx mori or other species of silkworm. The invention is also applicable to homologues of these proteins or recombinant proteins obtained by genetic engineering. The invention is further applicable to regenerated silk solutions prepared by dissolving silks in solutions containing chaotropic agents. More generally, it is thought that the invention is applicable to the storage of any proteins or protein analogues that are repetitive amphiphilic block co-polymers and which contain charged groups, although these materials are not limiting of the invention.

In the alkali storage compartment 40 several different types of alkaline solution can be used. For example, the alkali can be ammonia/acetic acid, ammonium acetate, ammonium/formic acid, or ammonium formate. These buffers are volatile and create in the protein storage compartment 30 an alkaline atmosphere. Tris/HCl, HEPES or PIPES can be used instead but these buffers are not volatile. In one embodiment of the invention, the alkaline buffer is selected from the group of alkalis consisting of ammonia, ammonium acetate, ammonium formate and ammonium citrate buffer. Potassium phosphate and potassium carbonate may also be suitable. In a preferred embodiment of the invention, the alkaline buffer contains 100-700 mM of calcium ions, preferably added in the form of calcium chloride.

In the preferred embodiment sodium azide is also added to the protein 20 in the first storage compartment in addition to the alkaline buffer. In an alternative embodiment of the invention, phenyl thiourea, sodium cyanide or potassium cyanide is added to the protein 20.

In another embodiment of the invention, shown in Fig. 2, the protein storage compartment 30 is separated from the alkali storage compartment 40 by means of a semi-permeable or porous membrane 60. The semi-permeable or porous membrane 60 allows the passage of ions to change the pH of the protein 20 stored in the protein storage compartment 30. In the event that a semi-permeable membrane is used, polyethylene glycol can be added to the alkaline buffer solution of up to 70% w/v to remove water from the protein solution in the protein storage compartment by reverse dialysis. Under these circumstances, the molecular weight of the polyethylene glycol used must be above the molecular weight cut-off of the semipermeable membrane. Other polymers can be used in this way provided that they are water soluble and are of sufficient size to prevent them from passing through the dialysis membrane.

Thus the protein 20 can be prevented from premature coagulation by treatment in the first compartment 30 for a period of time as short as one minute but preferably for periods of at least 20 minutes. This period of time depends on the quantity of the protein 20, its initial pH value, the temperature, the surface area of the protein 20 exposed to the alkaline buffer, the distance through which the alkaline buffer is required to diffuse to reach all of the protein 20 and the buffering capacities of the protein 20 and of the alkaline buffers.

In a preferred embodiment of the invention, the protein 20 is mixed with an alkaline buffer solution such as ammonium acetate or ammonium formate having a pH higher than 7.4 and a concentration equal to or greater than 0.1M. In the preferred embodiment of the invention, the alkaline buffer solution contains 100 to 700 mM of calcium ions and in excess of 0.0001 M sodium azide.

The suitability of different alkaline solutions for promoting the stability of the protein 20 was assessed in two ways: First, small drops of concentrated protein solutions were dialysed against different alkaline buffer solutions for time periods of up to four weeks and the gel or sol state of the protein solution determined at intervals. Typical results are

shown in Fig 3. Secondly, the validity of this approach was confirmed by attempting to spin fibres from small volumes of protein solution after storing them for different lengths of time in contact with alkaline buffers. In this second approach, a biomimetic spinning device of the general type described in PCT application No WO-A-01/38614 was used, the teachings of which are incorporated herein by reference. Tests with the biomimetic spinning device have shown that the protein 20 stored in contact with an atmosphere saturated with vapour derived from 1M ammonium hydroxide solution can still be spun to make a thread, fibre or filament after one week. The length of time for storage can be increased if sodium azide is added to the protein 20.

The apparatus and method can not only be used for storing natural and recombinant proteins, it may also be used to store regenerated solutions of fibroin and spidroin both of which have been prepared by dissolving silks made from this proteins in an appropriate solution containing a chaotropic (hydrogen bond breaking) agent. One example of such a chaotropic agent is a 50:50 v/v mixture of saturated lithium bromide and absolute ethanol.

Example 1: Extension of Storage Time

In the following example, the storage time is extended for protein solutions comprising silk worm protein obtained from the silk glands of Bombyx mori, regenerated Bombyx mori fibroin solution or concentrated spidroin solution obtained from the major ampullate glands of Nephila spiders.

In a first step the protein solution was transferred to a dialysis bag (MWCO 5-8 kDa) and concentrated by reverse dialysis against a solution containing 20% w/v PEG (MW 15-20 kDa) and 0.1 mM ammonium acetate puffer of pH 7.8 for five hours at 4°C.

The protein is gelled by dialysis against a solution containing 500 mM calcium chloride solution and 0.1 mM ammonium acetate buffer at pH 7.8 for one hour at 4°C. Sodium azide can be added to the dialysant to a final concentration of 0.001 mM to prevent bacterial growth.

The resulting gel can be stored at 4°C for at least four weeks.

The resulting gel can be converted back to a sol by dialysis against distilled water or aqueous 100 mM ethylene diamine tetracetic acid solution prior to extrusion or otherwise forming the object.

Example 2: Demonstration that fibroin is stored as a gel prior to spinning in a Bombyx mori silkworm.

The state of the fibroin in the posterior, middle and posterior part (glandular) of the anterior division of silk gland of the Bombyx mori silkworm was assessed by dissection under a binocular microscope at different stages in the silkworm's development. Glands rapidly removed from silkworms were transferred to silkworm Ringer solution (pH 7.8) for observation. The material in the lumen of the gland appears to be initially present as a sol at all stages up until the final instar a few days before cocoon spinning commences whereafter it is stored as a gel up to and during the initial stage of cocoon spinning. By cutting the duct (anterior part of the anterior division) across and watching whether the fibroin dope would flow out, it was demonstrated that the fibroin was present as a sol in this division immediately prior to and during spinning. Once spinning commences, sol formation appears to propagate progressively backwards through the silk dope as the size of the silk gland diminished during spinning. This demonstrates that gel formation was essential for safe storage of the silk and sol formation essential for the flow of silk dope down the duct for spinning.

Example 3: Effect of the addition and removal of calcium ions on the sol/gel state of the stored native fibroin

Solutions of fibroin dope were obtained by diluting the pooled contents of the middle division of the gland with 1 ml of 100 mM ammonium acetate buffer containing 10 mM sodium azide and 100 mM EDTA adjusted to pH 7.8 with concentrated ammonia solution or acetic acid. These solutions could be rapidly gelled by addition of 1volume of 1 M calcium chloride to 1 volume of the fibroin dope solution or by dialysis against 500 nM calcium chloride aqueous solutions. The protein could be returned to the sol state by dialysis against distilled water or 100 mM ammonium acetate buffer (pH 7.8) or more rapidly by dialysis against 100 mM ammonium acetate buffer (pH 7.8) containing 500 mM EDTA. This suggests that the sol /gel transition can be induced by the addition of

calcium ions and the gel can be caused to revert to the sol state by removing the calcium ions again. The calcium-induced transition appeared to be reversible after storage for days and indeed weeks in the gel condition.

Example 4: Effect of calcium ion addition or removal on the storage of native fibroin solutions.

A concentrated fibroin solution was obtained and gelled by the addition of 1 M calcium chloride as in described in Example 3. The length of time in which the gel could be stored stably in a state that could be turned into a sol by removal of calcium ions was tested. To do this samples of the gel that had been at 4° C for different lengths of time were taken and immediately dialysed against 100 mM ammonium acetate buffer (pH 7.8) containing 500 mM EDTA. These observations indicated that the calcium-fibroin gel could be safely transformed into a sol after storage for at least four weeks. In contrast the sol formed in the absence of calcium ions could only be stored for 5-8 days at 4°C before it formed a polymerised material which could not be converted to a sol even by the addition of further EDTA. Similar results were obtained using regenerated silk solution prepared by dissolving degummed silk in 9.6 M lithium bromide and dialysing the resultant solution in a low molecular weight cut of dialysis bag (Spectropor 10 KDa) against a solution containing 100 mM lithium, 20%w/v polyethylene glycol (nominal molecular weight 15KDa) . The gel prepared by adding calcium ions as above to this solution was however considerably less stiff than that obtained by gelling native fibroin.